GLOBAL CHANGE BIOLOGY
BIOENERGY

GCB Bioenergy (2016), doi: 10.1111/gcbb.12355

Evaluation of *Miscanthus sinensis* biomass quality as feedstock for conversion into different bioenergy products

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Abstract

Miscanthus is a promising fiber crop with high potential for sustainable biomass production for a biobased economy. The effect of biomass composition on the processing efficiency of miscanthus biomass for different biorefinery value chains was evaluated, including combustion, anaerobic digestion and enzymatic saccharification for the production of bioethanol. Biomass quality and composition was analyzed in detail using stem and leaf fractions of summer (July) and winter (March) harvested biomass of eight compositionally diverse *Miscanthus sinensis* genotypes. Genotype performance in tests for enzymatic saccharification, anaerobic digestion and combustion differed extensively. The variation between the best and the worst performing genotype was 18% for biogas yield (ml g^{-1} dm) and 42% for saccharification efficiency (glucose release as %dm). The ash content of the best performing genotype was 62% lower than that of the genotype with the highest ash content and showed a considerably high ash melting temperature during combustion. Variation between genotypes in biomass quality for the different thermochemical bioconversion processes was shown to be strongly correlated to differences in biomass composition. The most important traits that contributed favorably to biogas yields and saccharification efficiency were a high content of *trans*-ferulic acid, a high ratio of *para*-coumaric acid to lignin and a low lignin content. Additionally, a high content of hemicellulosic polysaccharides positively affected saccharification efficiency. Low contents of ash and inorganic elements positively affect biomass quality for combustion and low potassium and chloride contents contributed to a higher ash melting temperature. These results demonstrate the potential for optimizing and exploiting *M. sinensis* as a multipurpose lignocellulosic feedstock, particularly for bioenergy applications.

Keywords: anaerobic digestion, bioethanol, biogas, biomass quality, cell wall composition, combustion, enzymatic saccharification, lignin, *Miscanthus sinensis*

Received 28 October 2015; revised version received 29 February 2016 and accepted 29 February 2016

Introduction

Miscanthus is a promising fiber crop with high potential for sustainable biomass production in temperate climates (Heaton *et al*., 2010). It is a perennial C4 grass characterized by high annual biomass yields and a high resource-use efficiency (Long *et al*., 2001; Lewandowski *et al*., 2003b; Heaton *et al*., 2004, 2008; Van Der Weijde *et al*., 2013). Given its potential as a high yielding, low-input lignocellulosic feedstock, there is growing interest in the use of miscanthus biomass for a plethora of applications, in particular the production of bioenergy and biofuels (Brosse *et al*., 2012). Applications of lignocellulosic biomass are manifold, and three important bioenergy conversion routes include direct combustion, anaerobic digestion to produce biomethane and enzymatic saccharification and fermentation to produce bioethanol. The chemical composition and structure of cell walls play an important role in biomass quality for each of the aforementioned processes. Therefore, optimization of chemical composition and physical structure are envisioned to improve the process efficiency, which will subsequently contribute to the feasibility and economic success of bioenergy conversion technologies (Wyman, 2007; Torres *et al*., 2016).

There are different options to optimize and improve the biomass quality to facilitate the respective thermochemical conversion processes. Improved biomass quality can be achieved through breeding for

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quality traits. Currently, biomass quality is an important breeding objective in bioenergy crops such as miscanthus (Hodgson *et al.*, 2010; Van Der Weijde *et al.*, 2013). Another option is to improve biomass quality through 'on field quality management practices' such as fertilization and harvest time (Lewandowski & Kicherer, 1997; Lewandowski & Heinz, 2003; Lewandowski *et al.*, 2003a; Iqbal & Lewandowski, 2014). However, intrinsic differences between the distinct conversion routes result in route-specific requirements on biomass quality, either because they target other plant components or use another process to convert them into products. Lignin, for example, negatively affects the efficiency of biological conversion routes, such as fermentation or anaerobic digestion (Jørgensen *et al.*, 2007; Wyman, 2007; Zhao *et al.*, 2012), but has a favorable influence on the heating value of biomass for direct combustion (Lewandowski & Kicherer, 1997). Furthermore, for most bioconversion processes, the route-specific biomass quality requirements are not yet clearly defined due to a number of reasons. First of all, most bioconversion processes are not yet mature technologies and still need to be optimized. A second reason is that we do not yet fully understand the complex structure of lignocellulose and how it affects different bioconversion processes. The final reason is that biomass recalcitrance factors have evolved over a long time to protect the plant against environmental threats and we are now challenged to find ways to manipulate biomass recalcitrance without adversely affecting plant performance (Himmel *et al.*, 2007; Zhao *et al.*, 2012).

Improving biomass quality in miscanthus through plant breeding is plausible, as the genus *Miscanthus* harbors extensive genetic diversity that may be exploited for the development of new varieties (e.g., Clifton-Brown *et al.*, 2008; Heaton *et al.*, 2010). *Miscanthus sinensis* is one of the most promising species of miscanthus for biomass production in different environments, as it naturally occurs over a large geographical range in terms of latitude, longitude and altitude (Lewandowski *et al.*, 2000; Farrell *et al.*, 2006; Clifton-Brown *et al.*, 2008). Moreover, extensive variation in cell wall composition has been reported in *M. sinensis*, with cellulose content ranging from ~26 to 47%, hemicellulose content from ~25 to 43% and lignin content from ~5 to 15% of dry matter (Allison *et al.*, 2011; Qin *et al.*, 2012; Zhao *et al.*, 2014). Due to this extensive variation, the development of varieties with optimized biomass quality seems to be promising for various bioconversion routes.

The aim of this study was to understand how variation in lignocellulose composition affects the efficiency of different bioconversion processes and to explore the potential of miscanthus as a multipurpose crop that can be bred for a variety of different biobased applications. In this study, a diverse set of eight *M. sinensis* genotypes was selected from the miscanthus breeding program of Wageningen University and evaluated to gain insight in their potential for different biobased applications, including combustion, anaerobic digestion and enzymatic saccharification for ethanol production. The chemical composition of the stem and leaf fractions of biomass of these genotypes harvested in summer and winter was investigated to get insight in the effects of harvest time and genotype on traits considered to be relevant to the different bioenergy conversion technologies. The aim was to demonstrate the potential options for the use of miscanthus biomass as a feedstock for generating different types of bioenergy and to further define the selection criteria that will allow breeders to develop new varieties that are compositionally tailored to different value chains.

Materials and methods

Plant materials

Eight *M. sinensis* genotypes with a diverse cell wall composition profile were selected from the miscanthus breeding program of Wageningen University and used to establish a replicated field trial on a sandy soil in Wageningen, the Netherlands, in June 2013. A more detailed description and background information of the evaluated genotypes are given in Table S1. The genotypes were propagated *in vitro* to generate enough plantlets for setting up the trial. The trial was managed without irrigation, fertilization, pest, or weed control. The field trial had a design with four randomized blocks of eight plots. Plots had a size of 9 m^2 and contained 16 plants. All plots were surrounded by two rows with medium-sized *M. sinensis* plants to minimize possible border effects. Plant spacing between and within rows was 75 cm. In the establishment year, the trial was harvested (March 2014), but no samples were taken for analysis. After the establishment year, two different harvest regimes were imposed on the trial: Two of the four blocks were randomly assigned to be subjected to a double-cut harvest regime and the other two blocks were subjected to a single-cut harvest regime. The single-cut harvesting regime involved a cut in March 2015, referred to as 'winter cut'. The double-cut harvesting regime involved a green cut in mid-July 2014, referred to as 'summer cut' and a harvest of the regrowth in March 2015, referred to as 'regrowth cut', which coincided with the winter cut of the single-cut harvesting regime. At the time of the summer cut, genotypes OPM-42, 49 and 87 were already flowering, whereas the other genotypes were still in the vegetative phase.

For each of the three cuts, the leaf, stem and total dry matter yield were determined per plot after chopping the samples into ~2 cm chips and subsequent air drying at 60 °C for 72 h in a forced-air oven. The leaf fraction consisted only of leaf blades, with leaf sheets remaining in the stem fraction. The samples from the summer and the winter cut were subsequently used for laboratory analysis: The separated leaf and stem fractions were used for biochemical analysis of the different cell wall

 doi: 10.1111/gcbb.12355

components in both tissues, while a subsample in which stems and leaves were kept together was used for biomass quality assessment, including analyses of biogas yield, saccharification efficiency and combustion quality. All samples were ground using a hammer mill with a 1-mm screen.

Cell wall polymer composition

Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin contents (ADL) of stem dry matter were determined according to protocols developed by Ankom Technology (ANKOM Technology Corporation, Fairpoint, NY), which are essentially based on the work of Van Soest and coworkers (Van Soest, 1967; Goering & Van Soest, 1970). Neutral and acid detergent extractions were performed using an ANKOM 2000 Fiber Analyzer (ANKOM Technology Corporation). Acid detergent lignin was determined after 3-h hydrolysis of the ADF residue in 72% H_2SO_4 with continuous shaking. All fiber analyses were performed in triplicate. The weight fractions of detergent fiber residues in dry matter were subsequently used as estimate for the content of cell wall in dry matter (NDF%dm) and to obtain the contents of cellulose ([ADF%dm-ADL%dm]/NDF%dm × 100%), hemicellulosic polysaccharides ([NDF%dm-ADF%dm]/NDF%dm × 100%) and lignin (ADL%dm/NDF%dm × 100%) relative to the cell wall content.

Cell wall monosaccharide composition

The residual NDF material of the replicated fiber analyses was pooled per sample and used as a basis for determination of neutral sugar contents as described previously by Van Der Weijde *et al.* (2016). Measurements were taken in three replications. Briefly, 30 mg of NDF material was hydrolyzed in 0.3 ml 72% H_2SO_4 in a 10-ml glass pressure tube for 1 h at 30 °C with constant shaking (160 rpm). After 1 h, the acid concentration was diluted to 4% by adding 8.4 ml deionized water, after which samples were hydrolyzed for 3 h in a heating block set at 100 °C with a rotation speed of 160 rpm. After cooling down, the samples were centrifuged and a subsample of the supernatant was purified using a 0.45 μm filter. Contents of glucose (Glu), xylose (Xyl), arabinose (Ara) and galactose (Gal) in the purified supernatant were determined by high-performance anion exchange chromatography (HPAEC) analysis on a Dionex system equipped with a CarboPac PA1 column and a pulsed amperometric detector (Dionex, Sunnydale, CA). The degree of hemicellulose substitution (DHS) is the weight ratio of arabinose to xylose expressed as a percentage.

Monosaccharide acetylation

The amount of acetyl groups on monosaccharides was estimated by quantifying acetic acid in the undiluted, purified neutral sugar hydrolysate using an acetate dehydrogenase assay kit (Megazyme International Ireland Ltd., Bray, Ireland) adapted to a 96-well microplate format. The increase in sample absorbance at 340 nm following enzymatic dehydrogenase reactions was quantified using a Bio-Rad Microplate reader (Bio-Rad, Richmond, CA, USA).Acetic acid concentration in the sample was calculated from the increase in sample absorbance by interpolation from a six point standard curve of acetic acid (Megazyme International Ireland Ltd.). The degree of hemicellulose acetylation (DHA) is the dry weight of acetic acid expressed as a percentage of the dry weight of xylose on a sample basis.

Hydroxycinnamic acids

Hydroxycinnamic acids, specifically *p*-coumaric acid (*p*CA) and *trans*-ferulic acid (TFA), were quantified after extraction as described previously (Buanafina *et al.*, 2006). Briefly, an Eppendorf tube was filled with 10 mg NDF material of the samples and for the reference tests with 10 mg cellulose (Cellulose type 101; Sigma-Aldrich, Diegem, Belgium). The latter are also spiked with 100 μg TFA (Sigma-Aldrich) and *p*CA (Sigma-Aldrich). The tubes were subsequently incubated overnight in 750 μl 2 M NaOH at 25 °C and under constant shaking. Trimethoxycinnamic acid (TMCA, Sigma-Aldrich) was added as internal standard, and the pH of all samples was adjusted to two with HCL. A liquid–liquid extraction with diethyl ether was performed twice, after which the residue was dried for 1 h at 40 °C and resuspended in 1 ml 5% acetonitrile (MeCN) and vortexed for 15 s. Subsequently samples were 10 times diluted with 5% MeCN and stored at −20 °C before analysis. For each sample, 10 μl was injected into a liquid chromatography–high-resolution mass spectrometry (LC-HRMS) system. Chromatographic separation was performed with an Acquity Ultra Performance system (Waters, Milford, MA, USA) using a Waters BEH Shield C18 column (2.1 × 150 mm, 1.7 μm) held at 40 °C and equipped with an Shield C18 VanGuard precolumn (Waters). The mobile phase consisted of H_2O + 0.1% TFA (solvent A) and MeCN + 0.1% TFA (solvent B) at a flow rate of 0.35 ml min^{-1}. Gradient separation was performed as follows: linear increase from 5% to 50% B in 30 min, subsequent linear increase to 100% B in 1 min, held at 100% B for 6 min, followed by immediate decrease to 5% B and finally re-equilibration at 5% B for 5 min. Mass spectrometric detection and quantification were performed using a Synapt G2-S high-resolution mass spectrometer (Waters) acquiring full scan HRMS data (50–1200 Da) in resolution mode negative (20 000 FWHM). Source temperature and desolvation temperature were set 120 and 500 °C, respectively. Prior to analysis, the HRMS was calibrated (50–1200 Da) using a sodium formate solution. During analysis, leucine-enkephalin (200 pg μl^{-1}) was constantly infused as lock mass. Data were analyzed using the MassLynx software version 4.1 (Waters). The ratio of *p*CA to ADL (*p*CA/ADL) was calculated by expressing the dry weight of *p*CA as a percentage of the dry weight of ADL on a sample basis. Similarly, the ratio of TFA to xylose (DHF, for degree of hemicellulose feruloylation) was calculated by expressing the dry weight of TFA as a percentage of the dry weight of xylose on a sample basis.

Contents of ash and inorganic elements

Dried biomass samples were analyzed in the laboratory for N, Na, K, Ca, Mg, P, Cl, Si and ash content. Analysis of N was

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carried out following the Dumus principle using a Vario Macro cube (Elementaranalysensysteme GmbH, Hanau, Germany). For determination of Na, K and Ca, 500 mg dried biomass samples were dissolved in 8 ml HNO_3 (65%), to which 4 ml H_2O_2 was added to remove color. Samples were then digested in a microwave (Ethos.Lab, MLS GmbH, Leutkirch, Germany) at 120–180 °C and a pressure of 24.16 bar for 40 min. Digested samples were then filtered through Whatman filter paper and contents of P, K, Mg, Ca and Na in the extracts were determined using inductively coupled plasma-optical emission spectrometry (ICP-OES; Vista Pro, Varian Inc., Palo Alto, CA, USA). For determination of Cl, extractions were performed with hot deionized water and treated with a clarifying agent (Carrez I, containing 15 g $K_4Fe(CN)_6{\cdot}3H_2O$ in 100 ml deionized water and Carrez II, containing 30 g $Zn(CH_6COO)_2{\cdot}2H_20$ in 100 ml deionized water). The extracts were measured by ion chromatography (ICS 2000; Dionex Corporation, Sunnyvale, CA, USA). For the determination of Si content, samples were digested with HNO_3 and HF and measured with help of using ICP-OES (Vista Pro, Varian). Ash content was quantified gravimetrically after 4-h incineration in an electric muffle furnace at 550 °C.

Ash melting behavior during combustion

To assess the ash melting behavior during combustion process, the method was adopted from Tonn *et al.* (2012). Briefly, 100 mg ash samples were transferred to ceramic combustion boats (Lab Logistics group GmbH, Meckenheim, Germany) and subjected to four different combustion temperature treatments (800, 900, 1000 and 1100 °C) for 2 h in an electric muffle furnace. The electric muffle furnace was heated at an average rate of 10 °C min^{-1} until the required heating temperature was achieved. After 2 h, the combustion boats were transferred into an exicator to allow them to cool down before microscopic analysis. Each sample was analyzed under a stereo microscope (Zeiss Stemi 2000-C; Carl Zeiss AG, Oberkochen, Germany) at magnifications up to 40× and classified into one of four ash fusion classes (AFC) (Table 1) as described by Tonn *et al.* (2012).

Table 1 Ash fusion classes and ash fusion temperature along with microscopic observations (source: Tonn *et al.*, 2012)

Ash-fusion classes	Microscopic observations
(1) Loosening	Particles are arranged in loose layers, spatula can move through without any resistance, shiny surfaces with tiny molten vesicles
(2) Partially sintered	Particles start becoming compact through strong adhesive forces, still easy to disintegrate, produces crispy sound when spatula passes through, larger molten vesicles on the surface
(3) Highly sintered	Difficult to disintegrate, most of the area covered with larger molten vesicles. Organogenic material also visible in some parts
(4) Molten	Particles are completely molten, manual disintegration is not possible, no organogenic material visible

Biogas yield upon anaerobic digestion

The substrate-specific biogas (SSBY) and methane yields (SSMY) were measured in a biogas batch test under mesophilic conditions at 39 °C according to VDI guideline 4630. The biogas batch method was described by Kiesel & Lewandowski (2016) and certified after KTBL and VDLUFA inter-laboratory comparison test 2014. The fermentation period was 35 days. Four replicates of each sample were analyzed. A maize standard was analyzed alongside the miscanthus samples to monitor the activity of the inoculum. The inoculum originated from the fermenter of a commercial mesophilic biogas plant which uses the following substrates: maize silage, grass silage, cereal whole crop silage, liquid and solid cattle manure and small quantities of horse manure. The inoculum was sieved and diluted to 4% (w/w) dry matter with deionized water. Various macro- and micronutrients were added as described by Angelidaki *et al.* (2009). Afterward, the inoculum was incubated at 39 °C under anaerobic conditions for 6 days.

For the biogas batch analysis, 200 mg miscanthus samples were transferred into a 100-ml fermentation flask, 30 g inoculum was added, and the gas-containing headspace was flushed with nitrogen to attain anaerobic conditions. The fermentation flasks were closed gastight by a butyl rubber stopper and an aluminum cap. The pressure increase in the fermentation flasks was measured by puncturing the butyl rubber stopper with a cannula attached to a HND-P pressure meter (Kobold Messring GmbH, Hofheim, Germany). The biogas production was calculated as dry gas (water vapor pressure was considered) from the pressure increase and was standardized to 0 °C and 1013 hPa using Eqns (1) and (2). Equation (1) was used for the first measurement and considers the pressure increase due to warming from laboratory temperature to 39 °C and the water vapor partial pressure. Equation (2) was used for the subsequent 17 measurements, which were taken on regular basis.

$$V_{\text{biogas}} = V_{\text{HS}} * T_{\text{S}}/T_{\text{F}} * ((P_{\text{A1}} + P_{\text{F1}}) - (P_{\text{A0}} * T_{\text{F}}/T_{\text{Lab}}) - P_{\text{WP}})/P_{\text{S}} \quad (1)$$

where V_{biogas} = volume of produced biogas, V_{HS} = volume of gas-containing headspace, T_{S} = standard temperature of 273.15 °K (= 0 °C), T_{F} = fermentation temperature of 312.15 °K (= 39 C), P_{A1} = ambient pressure at first measurement, P_{F1} = overpressure in fermentation flasks at first measurement, P_{A0} = ambient pressure at sealing of the fermentation flasks (batch test start), T_{Lab} = laboratory temperature at sealing of the fermentation flasks (batch test start), P_{WP} = water vapor partial pressure at 39 C, P_{S} = standard pressure (1013 hPa)

$$V_{\text{biogas}} = V_{\text{HS}} * T_{\text{S}}/T_{\text{F}} * ((P_{\text{An}} + P_{\text{Fn}}) - (P_{\text{A}(n-1)} + P_{\text{F}(n-1)}))/P_{\text{S}} \quad (2)$$

where P_{An} = ambient pressure at the actual measurement, P_{Fn} = overpressure in fermentation flask at the actual measurement, $P_{\text{A}(n\text{-}1)}$ = ambient pressure at the previous

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time-point, $P_{F(n\text{-}1)}$ = overpressure in the fermentation flasks at the previous time-point.

During the course of the biogas batch test, it was occasionally necessary to remove the produced biogas from the fermentation flasks. The overpressure in the fermentation flasks was removed using a gastight syringe once it had reached an approximate value of 500 mbar. The biogas was transferred to a gastight storage flask where it was kept until the end of the batch test. After each gas collection, the remaining overpressure in the fermentation flasks was allowed to level off to ambient pressure by injecting a blank cannula. For the subsequent measurement, $P_{F(n\text{-}1)}$ was then set to zero in Eqn (2). At the end of the batch test, the remaining biogas in the headspace of the fermentation flasks was removed by active extraction with a syringe and also transferred into the storage flask. An aliquot of the collected biogas was used for analyzing the methane content using gas chromatography (GC-2014, Shimadzu, Kyoto, Japan). The gas chromatograph was equipped with a thermal conductivity detector, and the detection temperature was set to 120 °C. Two columns (HayeSep and Molsieve) were used for separation, with system temperature set at 50 °C and argon as carrier gas. The gas samples were injected using a Combi-xt PAL auto-sampler (CTC Analytics AG, Zwingen, Germany).

Saccharification efficiency for bioethanol production

Saccharification reactions were carried out as described previously by Van Der Weijde *et al.* (2016). Briefly, 500 mg biomass samples was briefly treated with α-amylase and repeatedly washed with deionized water (3×, 5 min, ~60°C) to remove all interfering soluble sugars. Remaining biomass was subjected to alkaline pretreatment with 15 ml 2% NaOH at 50°C and constant shaking (160 rpm) for 2 h in a shaker incubator (Innova 42; New Brunswick Scientific, Enfield, CT, USA). Pretreated samples were then washed to neutral pH with deionized water (2×, 5 min, 50°C) and with 0.1 M sodium citrate buffer (pH 4.6, 5 min, 50°C).

Saccharification reactions were subsequently carried out according to the NREL Laboratory Analytical Procedure 'Enzymatic saccharification of lignocellulosic biomass' (Selig *et al.*, 2008). Pretreated samples were hydrolyzed for 48 h with 300 μl of the commercial enzyme cocktail Accellerase 1500 (DuPont Industrial Biosciences, Leiden, the Netherlands) supplemented with 15 μl endo-1,4-β-xylanase M1 (Megazyme, Bray, IE, USA) in a shaker incubator (Innova 42; New Brunswick Scientific) set at 50°C and constant shaking (160 rpm). These enzymes combined have the following specific activities: endoglucanase 2200–2800 CMC U g^{-1}, beta-glucosidase 450–775 pNPG U g^{-1} and endoxylanase 230 U mg^{-1}. Reactions were carried out in 44 ml 0.1 M sodium citrate buffer (pH 4.6), containing 0.4 ml 2% sodium azide to prevent microbial contamination.

Enzymatic saccharification liquors were analyzed for glucose and xylose content by HPAEC as described previously for neutral sugars. The potential of a genotype for bioethanol production was assessed by expressing the total fermentable sugar yield in two ways. The first is the absolute yield of glucose and xylose as a percentage of dry matter (glucose release %dm and xylose release %dm). The second way is to express the yield of glucose and xylose as a percentage of the respective total available cell wall glucan (glucose conversion %) and xylan (xylose conversion %), as measures of saccharification efficiency.

Statistical analyses

General analyses of variance (ANOVA) were performed to determine the significance of genotype differences ($P < 0.05$) in compositional traits and quantitative route-specific quality parameters. Friedman's nonparametric ANOVA was performed to determine the significance of genotype differences in ash fusion classes. Variance analyses were performed following the standard procedure of a mixed effect model with a random genetic effect and a fixed block effect, following the model (3):

$$Y_{ij} = \mu + G_{ij} + B_j + e_{ij} \quad (3)$$

where Y_{ij} is the response variable, μ is the grand mean, G_{ij} is the genotype effect, B_j is the block effect, and e_{ij} is the residual error.

Correlation analyses were performed to identify the significance, strength and direction of interrelationships between traits using Pearson's correlation coefficients. Multiple linear regression analyses were performed for the development of simple regression equations for biogas yield and saccharification efficiency. All statistical analyses were performed using Genstat for Windows, 18th edition software package (VSN International, Hemel Hempstead, UK).

Results

Large differences in field performance between genotypes and harvest regimes

The field performance of the eight miscanthus genotypes was evaluated by assessing dry stem, leaf and total biomass yields of the genotypes from a single- and a double-cut harvest regime (Table 2). Biomass yields from the double-cut harvest regime were significantly lower than from the single-cut harvest regime. Averaged over all genotypes, the summer cut yielded 1803 kg dm ha^{-1}, and the regrowth cut yielded an additional 630 kg dm ha^{-1}. The winter cut, however, yielded on average 6314 kg dm ha^{-1}. The highest yielding genotype (OPM-69) in the winter cut had an average total biomass yield as high as 10 583 kg dm ha^{-1}. Furthermore, roughly 60% of the summer cut and roughly 45% of the regrowth cut consisted of stem material, whereas the biomass of the winter cut consisted almost completely of stem material. The genotypic variation for dry biomass yield and stem fraction of total yield, respectively, as realized during the first whole growing season was extensive (Table 2).

Genotypes show highly diverse cell wall composition

The summer cut and winter cut biomass samples of the eight miscanthus genotypes were analyzed

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Table 2 Means of a diverse set of eight *Miscanthus sinensis* accessions for total dry matter yield and the weight distribution of total dry matter among stem and leaf fractions evaluated in a single cut and double cut harvest regime following the first complete growing season

			Accession										
Harvest regime	Trait	Unit	OPM 42	OPM 48	OPM 49	OPM 65	OPM 69	OPM 73	OPM 77	OPM 87	Average	Range	*F*-prob.
Double-cut	Yield	kg dm ha^{-1}	747	490	423	614	994	1099	661	568	700	675	<0.001
(Summer cut)	Stem	%	60	53	63	52	70	66	58	58	60	18	<0.001
	Leaf	%	40	47	37	48	30	34	42	42	40	18	<0.001
Double-cut	Yield	kg dm ha^{-1}	1866	2649	1102	1664	2206	2427	1873	2329	2015	1548	0.006
(Regrowth cut)	Stem	%	44	54	56	43	46	55	70	44	52	27	0.022
	Leaf	%	56	46	44	57	54	45	30	56	48	27	0.022
Single-cut	Yield	kg dm ha^{-1}	5788	5948	4975	5422	11 759	7925	7494	6809	7015	6783	0.005
(Winter cut)	Stem	%	93	96	92	97	94	91	94	88	93	9	0.056
	Leaf	%	7	4	8	3	6	9	6	12	7	9	0.056

Table 3 Means of a diverse set of eight *Miscanthus sinensis* genotypes for stem biomass and cell wall components of the summer cut

		Genotype										
Trait	Unit	OPM 42	OPM 48	OPM 49	OPM 65	OPM 69	OPM 73	OPM 77	OPM 87	Average	Range	*F*-prob.
NDF	%dm	83.92	83.10	80.81	80.00	80.33	82.06	83.54	81.47	81.90	3.91	0.005
ADF	%dm	49.22	50.73	48.44	47.51	55.39	51.34	50.72	48.76	50.26	7.88	0.003
CEL	%cw	51.11	54.85	53.51	53.40	58.84	55.63	53.82	52.45	54.20	7.73	<0.001
HEM	%cw	41.34	38.95	40.06	40.63	31.04	37.43	39.29	40.14	38.61	10.31	<0.001
ADL	%cw	7.55	6.20	6.42	5.97	10.12	6.94	6.89	7.41	7.19	4.15	0.003
Glu	%cw	51.82	52.29	53.93	53.00	53.37	55.26	51.69	51.33	52.84	3.93	0.195
Xyl	%cw	32.02	31.30	33.07	30.84	27.54	30.24	31.62	30.89	30.94	5.53	0.005
Ara	%cw	3.43	3.52	3.78	3.36	2.57	2.73	3.44	3.28	3.26	1.21	0.002
Gal	%cw	0.38	0.27	0.33	0.31	0.24	0.20	0.37	0.23	0.29	0.19	<0.001

NDF, neutral detergent fiber; ADF, Acid detergent fiber; Cel, Cellulose; Hem, Hemicellulose; ADL, Acid detergent lignin; Glu, Glucose; Xyl, Xylose; Ara, Arabinose.

biochemically (Tables 3 and 4, respectively). The tables show the mean performance of each genotype for a wide set of stem biomass and cell wall traits, such as the content, chemical composition and structural complexity of various cell wall polymers. Significant differences between genotypes were found for nearly all cell wall components. Stem samples of the winter cut were analyzed in most detail, as they represent the largest weight fraction of all the harvested biomass.

In the summer cut, approximately 82% of the stem dry matter consisted of cell wall material, which increased to approximately 92% in the winter cut (Tables 3 and 4). In the winter cut, very little variation in stem cell wall content existed and genotypes were not found to be significantly different from each other (Table 4). The composition of the cell wall material also differed markedly between the summer and winter cut samples, with the summer cut samples generally being lower in cellulose and lignin contents, but higher in contents of hemicelluloses. In both cuts, particularly, large genotypic variation was found for Hem and ADL. Hemicellulose content in stem cell walls varied among genotypes ~31–41% in the summer cut and from ~29 to 37% in the winter cut. For stem cell wall, lignin content variation among genotypes ranged from ~6 to 10% in the summer cut and from ~8 to 13% in the winter cut (Tables 3 and 4).

In both cuts, also large variation was found in the neutral sugar composition of the stem cell wall material, particularly for arabinose and galactose, which are sugars that are present in side chains on the xylan backbone of grass hemicelluloses (Tables 3 and 4). For the winter cut stem samples, additional measurements were taken to investigate minor components of the cell wall matrix, such as hydroxycinnamic acids (TFA and *p*CA) and acetic acid. The ratios of arabinose to xylose (DHS), TFA to xylose (DHF), acetic acid to xylose (DHA) and *p*CA to ADL were investigated, as these provide

 doi: 10.1111/gcbb.12355

Table 4 Means of a diverse set of eight *Miscanthus sinensis* genotypes for stem biomass and cell wall components of the winter cut

		Genotype										
Trait	Unit	OPM 42	OPM 48	OPM 49	OPM 65	OPM 69	OPM 73	OPM 77	OPM 87	Average	Range	*F*-prob.
NDF	% dm	92.01	90.51	90.91	91.56	93.12	91.18	90.83	91.77	91.49	2.62	0.193
ADF	% dm	58.39	58.61	59.20	57.68	66.55	61.75	58.83	59.39	60.05	8.87	0.002
CEL	% cw	53.99	55.71	56.44	55.03	58.31	58.87	55.12	54.35	55.98	4.88	0.006
HEM	% cw	36.53	35.24	34.88	37.01	28.54	32.28	35.23	35.29	34.38	8.47	<0.001
ADL	% cw	9.47	9.05	8.68	7.96	13.15	8.86	9.65	10.36	9.65	5.19	<0.001
PCA	μg mg^{-1} cw	14.98	16.48	16.16	15.45	15.15	13.75	15.96	15.51	15.43	2.74	<0.001
PCA/ADL	% ADL	15.81	18.21	18.62	19.40	11.53	15.52	16.56	14.97	16.33	7.87	<0.001
Glu	% cw	46.20	46.33	45.32	45.30	48.30	49.09	45.03	46.65	46.53	4.07	0.040
Xyl	% cw	32.06	30.66	30.49	31.13	26.28	30.64	31.35	31.42	30.50	5.77	<0.001
Ara	% cw	3.18	3.21	3.08	3.00	2.41	2.44	3.11	2.81	2.91	0.80	0.001
Gal	% cw	0.38	0.25	0.26	0.28	0.22	0.16	0.34	0.23	0.26	0.22	<0.001
TFA	μg mg^{-1} cw	4.97	5.60	5.98	5.68	3.70	5.03	5.65	4.98	5.20	2.28	<0.001
Acetic acid	μg mg^{-1} cw	0.28	0.27	0.26	0.25	0.25	0.26	0.25	0.28	0.26	0.03	0.069
DHS	% Xyl	9.94	10.46	10.10	9.65	9.18	7.98	9.93	8.94	9.52	2.48	0.016
DHA	% Xyl	0.09	0.09	0.09	0.08	0.10	0.08	0.08	0.09	0.09	0.02	0.062
DHF	% Xyl	1.43	1.65	1.78	1.67	1.31	1.50	1.64	1.45	1.55	0.47	<0.001

NDF, neutral detergent fiber; ADF, Acid detergent fiber; Cel, Cellulose; Hem, Hemicellulose; ADL, Acid detergent lignin; PCA, para-coumaric acid; Glu, Glucose; Xyl, Xylose; Ara, Arabinose; TFA, trans-ferulic acid; DHS, Degree of hemicellulose substitution (ratio of arabinose to xylose); DHA, Degree of hemicellulose acetylation (ratio of acetic acid to xylose); DHF, Degree of hemicellulose feruloyation (ratio of TFA to xylose).

indications of the complexity and level of substitutions/side groups on xylose and lignin residues. Significant genotypic differences were found for all these ratios, with the exception of DHA, indicating that genetic variation for these trait ratios is available in the species (Table 4).

Leaf samples of the summer and winter cuts were also analyzed biochemically and the results are summarized in boxplots that display the variation in cell wall, cellulose, hemicellulose and lignin contents, respectively (Fig. 1a–d). Compared to the stem samples, leaf samples generally contained lower contents of NDF and cellulose, but higher contents of hemicellulosic polysaccharides. In the summer cut samples, stem tissues were higher in lignin content than leaf tissues, while in the winter cut samples, the opposite trend was observed (Fig. 1d).

Large variation in genotype performance in different bioenergy conversion processes

The potential as feedstock of eight *M. sinensis* genotypes for three different types of bioenergy conversion processes, that is, anaerobic digestion, enzymatic saccharification and combustion, was evaluated in this study. Genotype means of specific quality characteristics relevant to the different types of bioenergy conversion route are presented in Tables 5 and 6, for biomass samples harvested in the summer and winter cut, respectively. Genotypes showed significant differences for many specific quality traits relating to the different bioenergy applications. Anaerobic digestion of samples from the summer cut resulted in higher biogas yields compared to biomass samples from the winter cut, with genotype means for substrate-specific biogas yields ranging from 539 to 591 ml g^{-1} dry matter for the summer cut and 441 to 520 ml g^{-1} dry matter for the winter cut. Methane content in the produced biogas was approximately 52%, regardless of the time of harvest. The highest biogas yields were achieved by OPM-65 in the summer cut and OPM-73 in the winter cut, while in both cuts OPM-69 consistently had the lowest biogas yields.

To assess the quality of the biomass samples from the summer and the winter cut for fermentation of structural sugars into bioethanol, the samples were pretreated and incubated with a commercial enzyme cocktail to study the yield and efficiency of the release of fermentable sugars. Significant differences among genotypes were found for glucose release and glucose conversion in both harvests and for xylose release in the green cut, but not for xylose conversion (Tables 5 and 6), despite large differences between genotypes in hemicellulose content (Tables 3 and 4). Similar to the results for biogas yield, higher sugar release and saccharification efficiency were found using the biomass samples of

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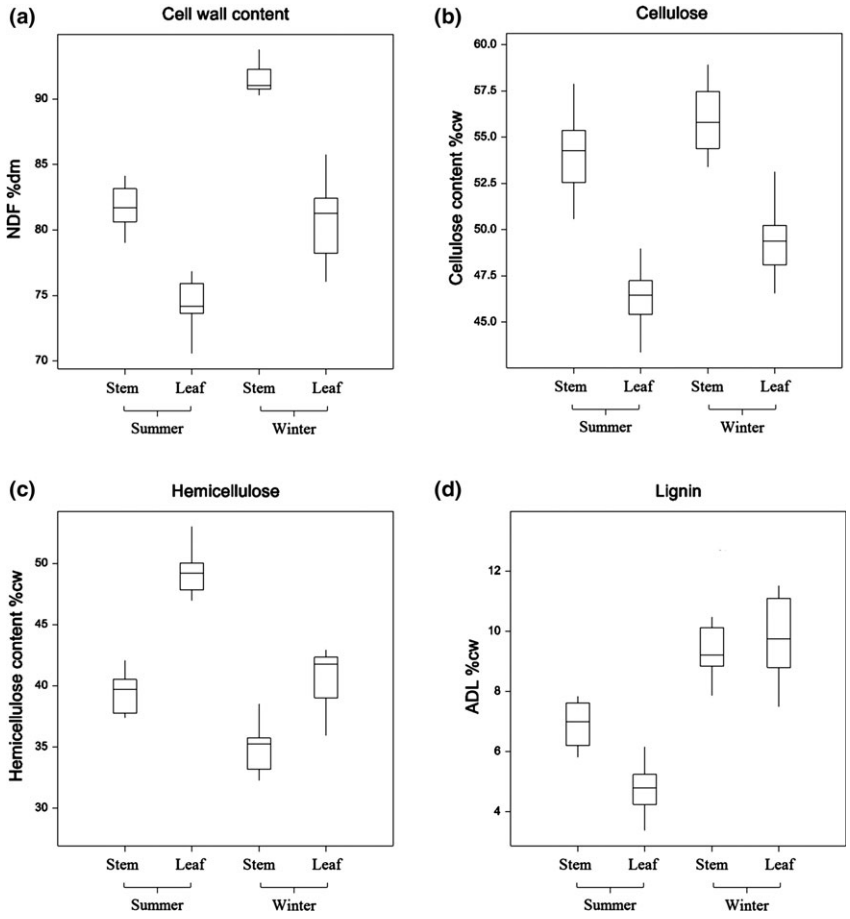

Fig. 1 Boxplots depicting variation in the cell wall content (a), cellulose (b), hemicellulose (c) and lignin (d) contents of miscanthus stem and leaf fractions of eight *Miscanthus sinensis* accessions harvested in a summer cut (July) or a winter cut (March).

the summer cut compared to the samples of the winter cut. Variation among genotypes in glucose conversion was extremely large, especially for biomass samples from the winter cut, ranging from 33% (for OPM-69) to 50% (for OPM-65).

For combustion quality, ash content is an important biomass quality determinant. The average ash content of the samples was 1.54% of dry matter in biomass from the winter cut compared to 3.28% of dry matter in biomass from the summer cut, when the plants had not yet senesced. As a result of the lower ash content, the quality of the biomass samples for combustion was higher in the winter cut than in the summer cut. Significant differences between genotypes for ash content were only found for biomass samples from the summer cut. Genotypes also showed significant differences in the contents of silicon and potassium in the summer cut and chloride and potassium in the winter cut. Furthermore, microscopic observations of ash melting behavior at different combustion temperatures were performed to make a classification of the genotypes into different ash fusion classes. Although samples could be assigned to distinct classes at each of the different temperatures, the classification for none of the tested temperatures has proven to lead to significant differences among genotypes (Tables 5 and 6).

Influence of biomass composition on genotype performance in different types of bioenergy conversion processes

The interrelations between compositional characteristics and specific quality traits for the different bioconversion processes were assessed using correlation analysis. Some of the most important correlations were highlighted in Fig. 2a–d, while the full correlation matrix is presented in Fig. 3. Similarities were found in the traits affecting the efficiency of enzymatic saccharification and anaerobic digestion. Both were negatively correlated to ADL and positively correlated to *p*CA/ADL and TFA (Fig. 2a–c). Additionally, both traits were negatively correlated to NDF and positively correlated to DHF (Fig. 3). No significant correlations were found between

Table 5 Mean performance of biomass of eight *Miscanthus sinensis* genotypes from a summer cut for quality traits relevant for specific bioenergy conversion routes

Route-specific quality characteristics	Genotype								Average	Range	*F*-prob.
	OPM 42	OPM 48	OPM 49	OPM 65	OPM 69	OPM 73	OPM 77	OPM 87			
Anaerobic digestion											
SSBY (ml g^{-1} dm)	562.90	572.87	575.40	591.78	538.84	572.20	561.01	560.33	566.92	52.94	<0.001
SSMY (ml g^{-1} dm)	290.30	296.50	296.64	305.34	278.19	293.57	288.39	290.37	292.41	27.15	<0.001
Methane (% SSBY)	52.13	52.25	52.11	52.13	52.18	51.94	52.02	52.30	52.13	0.36	0.203
Relative quality rating*	−	+	+	++	−−	+	−	−			
Fermentation											
Glucose release (%dm)	23.70	24.17	26.25	25.84	25.64	24.96	25.55	25.43	25.19	2.55	0.018
Xylose release (%dm)	6.70	6.85	7.28	7.27	7.71	7.25	7.09	6.82	7.12	1.01	0.175
Glucose conversion (%)	62.96	64.82	66.62	67.59	64.92	64.17	67.95	66.38	65.68	4.99	0.014
Xylose conversion (%)	30.84	32.24	32.48	32.68	33.13	30.95	32.65	31.38	32.04	2.30	0.409
Relative quality rating*	−	++	+	++	−−	+	−	−			
Combustion											
Ash (%dm)	2.59	5.05	3.27	3.38	3.42	2.38	3.00	3.13	3.28	2.67	0.006
Silicon (Si) (%dm)	0.31	0.44	0.26	0.33	0.29	0.18	0.26	0.34	0.30	0.26	0.046
Chloride (Cl) (%dm)	0.14	0.24	0.18	0.15	0.19	0.12	0.16	0.14	0.16	0.12	0.065
Potassium (K) (%dm)	0.84	1.90	1.28	1.15	1.42	0.95	1.13	1.17	1.23	1.07	<0.001
Calcium (Ca) (%dm)	0.18	0.21	0.15	0.16	0.09	0.28	0.18	0.18	0.18	0.19	0.670
AFC – 800 °C	1.00	3.00	3.00	1.00	1.75	1.00	1.50	1.75	1.75	2.00	0.066†
AFC – 900 °C	2.00	3.25	4.25	2.00	3.00	1.50	2.00	3.75	2.72	2.75	0.130†
AFC – 1000 °C	3.50	4.25	4.50	4.50	3.50	2.00	4.50	4.25	3.88	2.50	0.088†
AFC – 1100 °C	5.00	4.50	5.00	5.00	4.50	3.75	5.00	5.00	4.72	1.25	0.195†
Relative quality rating*	+	−−	−	−	−	++	+	+			

SSBY, substrate-specific biogas yield; SSMY, substrate-specific methane yield.

*Rating based on ranking genotypes by SSBY for anaerobic digestion, by glucose yield for fermentation and by HHV for combustion route. Rank 1 scored '++', rank 2–4 scored '+', rank 5–7 scored '−' and rank 8 scored '−−'.

†*P*-value using chi-square approximation resulting from Friedman's nonparametric ANOVA test.

biogas yield and saccharification efficiency, but a weak correlation was found between biogas yield and glucose release. Some cell wall compositional traits were not correlated to biogas yield, but did show correlations to the release and yield of glucose and xylose. Such correlations included positive correlations with Hem, Xyl, Ara and Gal, and negative correlations with ADF, Cel and Glu (Figs 2d and 3).

Multiple regression analysis was performed to develop regression models for glucose conversion and biogas yield based on cell wall compositional characteristics. A simple regression model was found for glucose conversion including only two traits, *p*CA/ADL and galactose, which cumulatively explained 83.2% of the variation for glucose conversion among these genotypes. Two simple regression models were found for SSBY, one which included ADL and galactose, and a second which included *p*CA/ADL and arabinose. Both models were able to account for 83.4% of the variation for SSBY among these genotypes.

Only two cell wall compositional characteristics were found to be correlated to combustion specific quality traits, that is, *p*CA content ($r = 0.68$) and DHS ($r = 0.54$), which both showed a positive correlation to the classification of samples to ash fusion classes at a combustion temperature of 800 °C (Fig. 3). However, inorganic elements silicon, potassium and calcium were strongly positively correlated to ash formation during combustion. Moreover, potassium and chloride were shown to be significantly correlated to classification of the genotypes in different ash fusion classes at all tested combustion temperatures (Table 7).

Discussion

Large genetic diversity in biomass composition and quality

The extensive genetic diversity in cell wall compositional traits found in the eight *M. sinensis* genotypes analyzed in this study indicate that there is a large potential in this species for the improvement of biomass quality for different applications. Particularly large variation between genotypes was found for the contents of

 doi: 10.1111/gcbb.12355

Table 6 Mean performance of biomass of eight *Miscanthus sinensis* genotypes from a winter cut for quality traits relevant for specific bioenergy conversion routes

	Genotype										
Route-specific quality characteristics	OPM 42	OPM 48	OPM 49	OPM 65	OPM 69	OPM 73	OPM 77	OPM 87	Average	Range	*F*-prob.
Anaerobic digestion											
SSBY (ml g^{-1} dm)	457.89	500.75	502.08	507.83	441.15	520.08	473.11	462.90	483.22	78.93	0.01
SSMY (ml g^{-1} dm)	236.61	256.40	258.59	260.77	228.70	266.57	244.52	238.11	248.78	37.86	0.018
Methane (% SSBY)	52.27	51.94	52.13	52.02	52.39	51.95	52.27	52.12	52.14	0.46	0.013
Relative quality rating*	–	+	+	+	–	++	–	–			
Fermentation											
Glucose release (%dm)	18.63	18.66	19.68	20.78	14.64	18.25	18.67	17.00	18.29	6.14	0.007
Xylose release (%dm)	8.41	7.74	7.88	8.25	5.78	7.37	8.12	7.66	7.65	2.62	0.005
Glucose conversion (%)	43.83	44.48	47.74	50.11	32.55	40.77	45.65	39.77	43.11	17.57	0.003
Xylose conversion (%)	29.29	28.59	29.12	29.62	24.35	27.22	29.31	27.44	28.12	5.27	0.077
Relative quality rating*	+	–	+	++	–	–	+	–			
Combustion											
Ash (%dm)	1.67	1.77	1.54	1.09	1.64	1.45	1.62	1.56	1.54	0.68	0.358
Silicon (Si) (%dm)	0.37	0.35	0.36	0.30	0.36	0.34	0.35	0.37	0.35	0.07	0.993
Chloride (Cl) (%dm)	0.02	0.06	0.00	0.01	0.05	0.03	0.03	0.02	0.03	0.06	0.002
Potassium (K) (%dm)	0.07	0.28	0.06	0.04	0.18	0.10	0.11	0.04	0.11	0.24	0.001
Calcium (Ca) (%dm)	0.10	0.10	0.11	0.08	0.08	0.09	0.10	0.11	0.10	0.03	0.531
AFC – 800 °C	1.00	2.75	1.50	1.00	1.50	1.00	1.75	1.25	1.47	1.75	0.076†
AFC – 900 °C	1.50	3.25	1.50	1.25	2.00	1.75	2.00	1.50	1.84	2.00	0.076†
AFC – 1000 °C	1.75	4.25	2.00	2.00	2.25	2.25	2.50	2.25	2.41	2.50	0.254†
AFC – 1100 °C	2.25	5.00	2.50	2.25	2.50	2.75	3.00	2.75	2.88	2.75	0.277†
Relative quality rating*	–	–	+	++	–	+	–	+			

SSBY, substrate-specific biogas yield; SSMY, substrate-specific methane yield.

*Rating based on ranking genotypes by SSBY for anaerobic digestion, by glucose yield for fermentation and by ash content for combustion route. Rank 1 scored '++', rank 2–4 scored '+', rank 5–7 scored '–' and rank 8 scored '–'.

†*P*-value using chi-square approximation resulting from Friedman's nonparametric ANOVA test.

Hem and ADL, which are the key factors determining lignocellulose recalcitrance (Xu *et al.*, 2012; Torres *et al.*, 2014; Van Der Weijde *et al.*, 2016). Additionally, significant genotypic variation was found for specific traits that relate to the degree of cross-linking between hemicelluloses or between hemicelluloses and lignin, and more specifically to the degree of substitution of the xylan backbone of hemicellulosic polysaccharides by arabinose (DHS), the degree of xylan acetylation (DHA) and feruloyation (DHF), and the ratio of *para*-coumaric acid to lignin (*p*CA/ADL). This is an important observation, as there is strong evidence that cell wall cross-links play important roles in cell wall degradability (e.g., Hatfield *et al.*, 1999; Grabber *et al.*, 2004; Torres *et al.*, 2014; De Souza *et al.*, 2015).

The performance of the genotypes in different bioenergy conversion processes was evaluated. These tests showed significant genotypic differences for many specific quality traits relating to anaerobic digestion and enzymatic saccharification. This finding indicates that considerable improvements in the techno-economic efficiency of bioconversion processes can be achieved by selecting a more suitable feedstock, as for example suggested for maize stover (Torres *et al.*, 2016). For enzymatic saccharification of winter harvested biomass, for example, the best performing genotype released 42% more glucose and 45% more xylose per gram dry matter than the worst performing genotype (Table 6). Similarly, for anaerobic digestion, the best performing genotype achieved 18% higher biogas yield than the worst performing genotype (Table 6). These findings indicate that major improvements in final product yield are possible, which will probably have a favorable effect on process economics. Also processing conditions may become less severe with a more suitable feedstock. The mild pretreatment reactions in saccharification experiments were not only chosen because they are optimal for the detection of genotypic differences in conversion efficiency, but also because they give information on the potential to reduce the severity of pretreatment conditions, while maintaining high yields of fermentable sugars. Savings with respect to energy and chemical consumption can be realized in this way,

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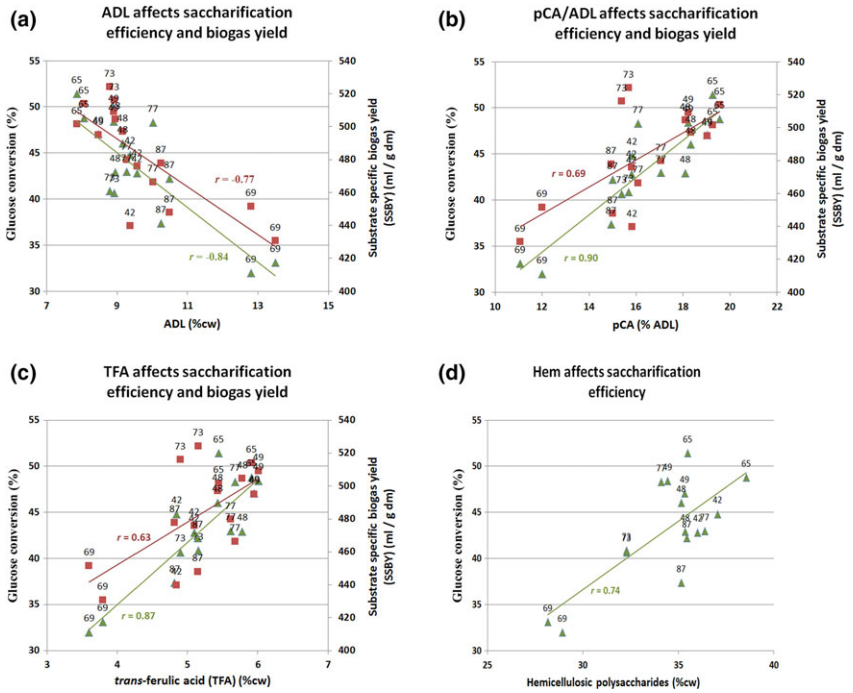

Fig. 2 The effects of cell wall compositional traits ADL (a), pCA/ADL (b), TFA (c) and Hem (d) on saccharification efficiency and biogas yield in stem samples of the winter cut. Saccharification efficiency was plotted as glucose conversion as a percentage of total cell wall glucan (green triangles), and biogas yield was plotted as substrate-specific biogas yield expressed in ml g^{-1} dm (red squares). Number labels represent accession numbers (OPM).

which will be a major cost reduction for the production of bioethanol.

Similarly, significant genotypic variation in contents of ash and inorganic elements was found, which can be exploited to improve the techno-economic performance of biomass combustion processes. Ash and certain inorganic elements are known to cause corrosion, slagging and fouling of the combustion chamber, thereby decreasing the quality of the biomass for combustion. Good combustion quality pertains to low ash content and a high ash melting point. Considerable genotypic variation in potassium and chlorine contents was found (Tables 5 and 6). This is in agreement with the large genotypic variation for elemental composition reported for *M. sinensis* (Atienza *et al*., 2003a,b). The classification of genotypes in ash fusion classes showed that the ashes of some genotypes (OPM-49 and OPM-65) were still only partly sintered at a combustion temperature of 1000 °C, whereas ashes of another genotype (OPM-48) at the same temperature were already completely molten (Tables 5 and 6). OPM-65 was shown to have a 62% lower ash content and was consistently classified in a lower ash fusion class during combustion than OPM-48, which is indicative of a higher ash melting point. For many important biochemical components and biomass quality traits, significant genotypic differences were found in this diverse set of *M. sinensis* genotypes that can potentially be exploited to optimize the feedstock for different applications.

Improving bioconversion efficiency by optimization of biomass composition

To show that genotype performance in bioconversion processes can be improved by optimizing biomass composition, correlation analyses were performed between compositional traits and biomass quality characteristics. It was shown that the efficiency of anaerobic digestion and saccharification is affected by biomass composition in a similar way. Lignin content had a negative impact on both conversion technologies, as anticipated and as is well established in literature (Campbell & Sederoff, 1996; Akin, 2008; Dandikas *et al*., 2014; Van Der Weijde *et al*., 2016). A high content of hemicellulosic polysaccharides was furthermore shown to be favorable for saccharification efficiency (r = 0.74, Figs 2d and 3).

Hemicellulosic polysaccharides and lignin both provide structural rigidity to the cell wall and are often negatively correlated (in this study $r = -0.77$, Fig. 3) (Qin *et al*., 2012; Torres *et al*., 2014; Van Der Weijde *et al*., 2016). Reductions in lignin content may be compensated for by an increase in hemicellulosic

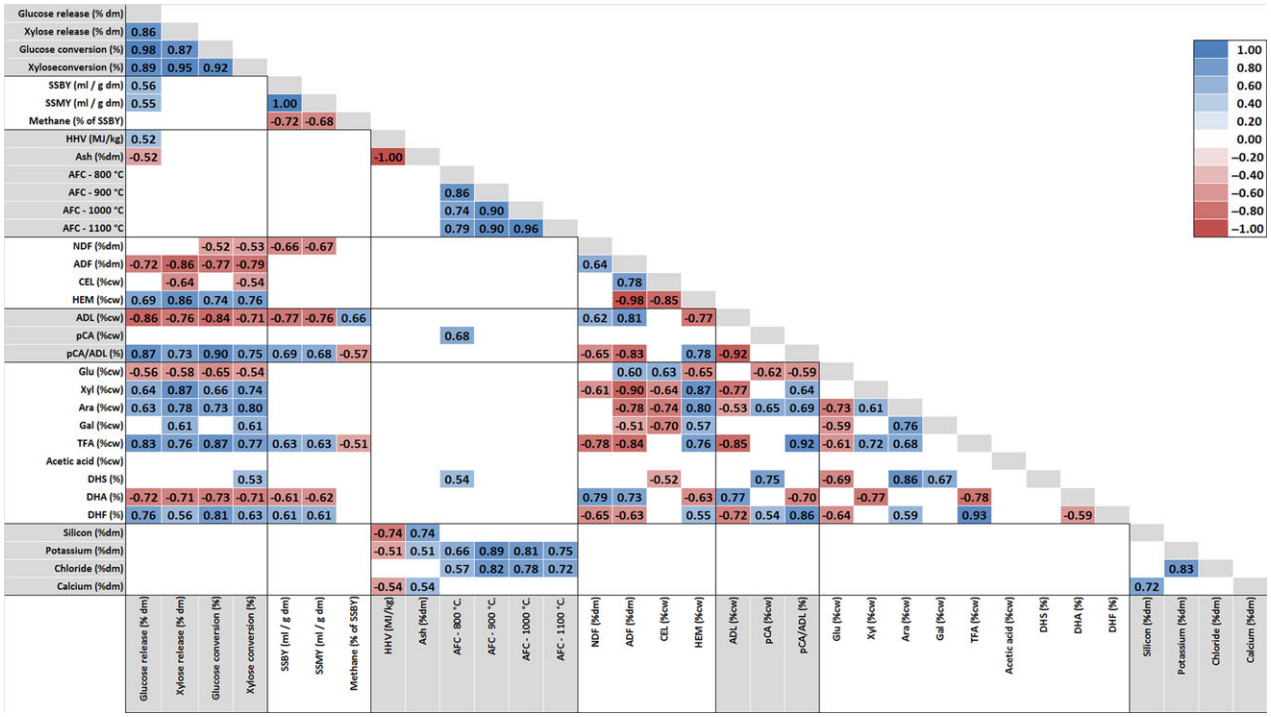

Fig. 3 Heat map depicting the extent and the direction of correlations among biomass compositional and biomass quality traits. Only Pearson correlation coefficients that differed significantly from zero ($P > 0.05$) are reported. Blue values indicate positive correlation coefficients, and red values indicate negative correlation coefficients.

Table 7 Impact of elemental composition on ash formation and ash melting behavior during combustion assessed using correlation analysis. Only Pearson correlation coefficients that differed significantly from zero ($P > 0.05$) are reported

Combustion specific quality traits	Silicon (% dm)	Potassium (% dm)	Chloride (% dm)	Calcium (% dm)
Ash (% dm)	0.74	0.51		0.54
AFC – 800 °C		0.66	0.57	
AFC – 900 °C		0.89	0.82	
AFC – 1000 °C		0.81	0.78	
AFC – 1100 °C		0.75	0.72	

polysaccharides, as well as in hemicellulose–hemicellulose and hemicellulose–lignin cross-links, so that lowering lignin content not necessarily leads to concomitant detrimental reductions in plant cell wall rigidity and associated negative effects on plant fitness. The accompanying changes in the cell wall matrix, however, while still imparting strength to the cell wall, might make the cell wall less recalcitrant to biological conversion processes, such as anaerobic digestion or enzymatic saccharification. This theory is supported by the fact that hemicelluloses are often found to be positively associated to cell wall degradability and saccharification efficiency (Xu *et al.*, 2012; Li *et al.*, 2013; Torres *et al.*, 2013).

Detailed profiling of the samples for minor cell wall components, such as acetic acid, *trans*-ferulic acid and *para*-coumaric acid, as well as hemicellulose monomeric constitution, was also proven to be important for understanding the effects of composition on biomass quality. The content of *trans*-ferulic acid was found to have a strong positive effect on the efficiency of both anaerobic digestion and enzymatic saccharification (Fig. 2c). In the literature, ferulate content is often considered to be negatively associated with cell wall degradability, as it is a key component that mediates cross-links between hemicelluloses and lignin (Hatfield *et al.*, 1999; Grabber, 2005; Yu *et al.*, 2005) and because feruloylated arabinose side chains of hemicelluloses are implicated as an initiation/nucleation site for lignin polymerization and deposition (Ralph *et al.*, 1995). However, it has also been reported that lignins that extensively incorporate hydroxycinnamic esters can be easily depolymerized using alkaline pretreatments (Ralph, 2010), which may help to explain the positive associations found in this study. Moreover, TFA content had a strong negative correlation ($r = -0.85$) to lignin content. Therefore, TFA content may be indirectly positively associated to biogas yield and saccharification efficiency.

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In addition, ratios between the different cell wall components were also found to be important, such as the ratio of *p*CA to ADL and the ratio of arabinose to xylose (DHS), which both positively affected biogas yield and saccharification efficiency (Figs 2b and 3). The positive effect of a higher ratio of arabinose to xylose is implicated to be due to a reduction in cellulose crystallinity associated with increase hemicellulose–cellulose cross-linking (Xu *et al*., 2012; Li *et al*., 2013). *p*CA is a phenolic compound that is ester-bound mainly to the S-subunit of the lignin polymer. A higher ratio of *p*CA to ADL might thus reflect a higher fraction of the lignin polymer to be comprised of the S-subunit. A higher S/G ratio is in literature sometimes associated with a higher saccharification efficiency (Li *et al*., 2010; Studer *et al*., 2011), especially with no or mild pretreatment (Chen & Dixon, 2007). It is also suggested that acylation of lignin with *p*CA impairs the copolymerizing of ferulates with monolignols (Grabber, 2005), which may also contribute to increased cell wall degradability. A high content of TFA, a high ratio of *p*CA to ADL and a low content of lignin are thus potentially interesting breeding targets for miscanthus for improving biomass quality for both saccharification and anaerobic digestion.

Although anaerobic digestion and enzymatic saccharification shared similar correlation patterns to compositional characteristics, the strength of these correlations was higher for saccharification efficiency, which indicates that this conversion process was more dependent on cell wall composition than biogas production. Moreover, biogas yield and saccharification efficiency were not significantly correlated to each other, suggesting that there are biomass quality traits that influence these conversion processes differently. One such trait was found to be Hem, which positively contributed to saccharification efficiency ($r = 0.74$, Figs 2d and 3), but not to biogas yield. Torres *et al*. (2014) showed that digestibility in rumen liquid (an anaerobic digestion process) and saccharification efficiency have many communalities, but a critical difference was that degree of hemicellulose substitution was relevant for saccharification efficiency, but not a major determinant for rumen liquid digestibility; a digestion process that resembles the process of anaerobic digestion for biogas production. This is also shown by the fact that the relative quality rating of the genotypes differed for anaerobic digestion and saccharification processes, with the best genotype for biogas production (OPM-73) being one of the worst for saccharification (Table 4). However, there were also genotypes that performed well in both platforms (for example, OPM-65), which indicates that it might be possible to improve biomass quality for both anaerobic digestion and enzymatic saccharification simultaneously.

For both conversion routes, it was clear that the summer cut had a better biomass quality than the winter cut, which is partly explained by the fact that lignin contents in the summer cut were much lower than in the winter cut. Lignin is mainly deposited after plant cells stop growing, when cell walls no longer need to accommodate cell expansion and become rigidified by lignification (Lam *et al*., 2013; Da Costa *et al*., 2014). Other factors that contributed to the higher conversion efficiencies of biomass of the summer cut are the facts that the relative weight ratio of leaves to total biomass was higher in the summer cut (Table 2), and that leaves were shown to have lower lignin contents in the summer cut then stem fractions (Fig. 1d). Despite higher conversion efficiencies, summer harvesting of miscanthus was shown to have a considerable and negative impact on total annual harvestable biomass yields, as the accumulated yield of the summer cut and the regrowth cut achieved only ±40% of the yield achieved in the winter cut (Table 2). Like for the genotypes evaluated in this study, a low tolerance to early green cuttings in July and August was also reported for *M. x giganteus*. However, a green harvest in October was shown to have less detrimental effects on crop yield, while beneficially affecting biomass quality for biogas production compared to winter harvesting (Kiesel & Lewandowski, 2016).

Combustion efficiency is known to be heavily dependent on the elemental composition of the feedstock, as such elements form ash in the combustion chamber, can be corrosive and cause slagging and fouling (Lewandowski & Kicherer, 1997; Atienza *et al*., 2003a,b). Not surprisingly, contents of inorganic elements and ash were much lower in samples from the winter cut than from the summer cut, as these elements are translocated into the roots during winter and removed from the plant by leaf shed (Lewandowski & Heinz, 2003; Lewandowski *et al*., 2003a). In addition, due to natural drying on the field during winter, the dried stems and leafs are more easily fractured by wind, which facilitates the leaching of inorganic elements in periods of rain. The low ash contents in samples of biomass from the winter cut compared to the corresponding samples from the summer cut favorable affect combustion quality (Tables 3 and 4). Moreover, it is known that lignin has a higher caloric value than cellulose and hemicellulose (Lewandowski & Kicherer, 1997) and samples harvested from the winter cut were shown to have higher lignin contents (Tables 3 and 4, Fig. 1d). Ash melting behavior could also be optimized. It was shown that potassium and chlorine were associated with lowering the ash melting point and that low contents of these elements positively affect combustion quality (Table 7). The relative quality rating of genotypes for combustion quality

 doi: 10.1111/gcbb.12355

differed for some genotypes from that for biogas or for saccharification, but notably there were as well genotypes that performed well in all conversion platforms, such as OPM-49 and OPM-65. However, these were not the highest yielding genotypes. The highest yielding genotype (OPM-69) on the other hand unfortunately tended to slag and had higher contents of Cl and K, resulting in a low quality for combustion. These results show that it is possible to optimize biomass quality for different utilization options simultaneously and develop multipurpose genotypes, but that several quality traits need to be cross-bred. Extensive genetic variation for many biomass quality traits was found in the eight *M. sinensis* genotypes evaluated in this study, but it is likely that the full extent of variation for these traits within the species is even broader. The exploitation of such variation through breeding will greatly accelerate the realization of biomass derived energy and fuel production, as well as many other biobased applications, generating many market options for the use of miscanthus biomass.

Acknowledgements

The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no 289159. We gratefully acknowledge the contributions of Kai Schwartz, Heike Meyer and Claire Lessa Alvim Kamei for bringing the genotypes into *in vitro* culture. We gratefully acknowledge the help of Arturo Chica Villa, Eben Haeser Sembiring, Juan Carlos Rivas, Dominik Ewald, Annemarie Dechesne, Eyup Oztutuncu, Verena Dobler, Dagmar Mezger and Els van Pamel for their technical assistance during laboratory analyses. We further acknowledge Genencor International B.V./DuPont Industrial Biosciences for kindly supplying us with their commercial Accellerase 1500 enzyme cocktail used in this study.

References

Akin DE (2008) Plant cell wall aromatics: influence on degradation of biomass. *Biofuels, Bioproducts and Biorefining*, **2**, 288–303.

Allison GG, Morris C, Clifton-Brown J, Lister SJ, Donnison IS (2011) Genotypic variation in cell wall composition in a diverse set of 244 accessions of Miscanthus. *Biomass and Bioenergy*, **35**, 4740–4747.

Angelidaki I, Alves M, Bolzonella D *et al.* (2009) Defining the biomethane potential (BMP) of solid organic wastes and energy crops: a proposed protocol for batch assays. *Water Science and Technology*, **59**, 927–934.

Atienza SG, Satovic Z, Petersen KK, Dolstra O, Martin A (2003a) Influencing combustion quality in *Miscanthus sinensis* Anderss.: identification of QTLs for calcium, phosphorus and sulphur content. *Plant Breeding*, **122**, 141–145.

Atienza SG, Satovic Z, Petersen KK, Dolstra O, Martín A (2003b) Identification of QTLs influencing combustion quality in *Miscanthus sinensis* Anderss. II. Chlorine and potassium content. *Theoretical and Applied Genetics*, **107**, 857–863.

Brosse N, Dufour A, Meng X, Sun Q, Ragauskas A (2012) Miscanthus: a fast-growing crop for biofuels and chemicals production. *Biofuels, Bioproducts and Biorefining*, **6**, 580–598.

Buanafina MMDO, Langdon T, Hauck B, Alton SJ, Morris P (2006) Maniplating the phenolic acid content and digestibility of italian ryegrass (*Lolium multiflorum*) by vacuolar-targeted epession of a fungal ferulic acid esterase. *Applied Biochemistry and Biotechnology*, **130**, 416–426.

Campbell MM, Sederoff RR (1996) Variation in lignin content and composition (mechanisms of control and implications for the genetic improvement of plants). *Plant Physiology*, **110**, 3.

Chen F, Dixon RA (2007) Lignin modification improves fermentable sugar yields for biofuel production. *Nature Biotechnology*, **25**, 759–761.

Clifton-Brown JC, Chiang YC, Hodkinson TR (2008) Miscanthus: genetic resources and breeding potential to enhance bioenergy production. In: *Genetic Improvement of Bioenergy Crops* (ed. Vermerris W), pp. 273–294. Springer Science+Business Media, LLC, New York, NY.

Da Costa RMF, Lee SJ, Allison GG, Hazen SP, Winters A, Bosch M (2014) Genotype, development and tissue-derived variation of cell-wall properties in the lignocellulosic energy crop Miscanthus. *Annals of Botany*, **114**, 1265–1277.

Dandikas V, Heuwinkel H, Lichti F, Drewes JE, Koch K (2014) Correlation between biogas yield and chemical composition of energy crops. *Bioresource Technology*, **174**, 316–320.

De Souza AP, Kamei CLA, Torres AF, Pattathil S, Hahn MG, Trindade LM, Buckeridge MS (2015) How cell wall complexity influences saccharification efficiency in *Miscanthus sinensis*. *Journal of Experimental Botany*, **66**, 4351–4365.

Farrell AD, Clifton-Brown JC, Lewandowski I, Jones MB (2006) Genotypic variation in cold tolerance influences the yield of Miscanthus. *Annals of Applied Biology*, **149**, 337–345.

Goering HK, Van Soest PJ (1970) *Forage Fiber Analyses (Apparatus, Reagents, Procedures, and Some Applications)*. Agricultural Handbook No. 379. Agricultural Research Service - United States Department of Agriculture, Washington, DC.

Grabber JH (2005) How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies. *Crop Science*, **45**, 820–831.

Grabber JH, Ralph J, Lapierre C, Barrière Y (2004) Genetic and molecular basis of grass cell-wall degradability. I. Lignin–cell wall matrix interactions. *Comptes Rendus Biologies*, **327**, 455–465.

Hatfield RD, Ralph J, Grabber JH (1999) Cell wall cross-linking by ferulates and diferulates in grasses[1]. *Journal of the Science of Food and Agriculture*, **79**, 403–407.

Heaton E, Voigt T, Long SP (2004) A quantitative review comparing the yields of two candidate C4 perennial biomass crops in relation to nitrogen, temperature and water. *Biomass and Bioenergy*, **27**, 21–30.

Heaton EA, Dohleman FG, Long SP (2008) Meeting US biofuel goals with less land: the potential of Miscanthus. *Global Change Biology*, **14**, 2000–2014.

Heaton EA, Dohleman FG, Miguez AF *et al.* (2010) Miscanthus. A promising biomass crop. *Advances in Botanical Research*, **56**, 76–137.

Himmel ME, Ding S-Y, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science*, **315**, 804–807.

Hodgson EM, Lister SJ, Bridgwater AV, Clifton-Brown J, Donnison IS (2010) Genotypic and environmentally derived variation in the cell wall composition of Miscanthus in relation to its use as a biomass feedstock. *Biomass and Bioenergy*, **34**, 652–660.

Iqbal Y, Lewandowski I (2014) Inter-annual variation in biomass combustion quality traits over five years in fifteen Miscanthus genotypes in south Germany. *Fuel Processing Technology*, **121**, 47–55.

Jørgensen H, Kristensen JB, Felby C (2007) Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. *Biofuels, Bioproducts and Biorefining*, **1**, 119–134.

Kiesel A, Lewandowski I (2016) Miscanthus as biogas substrate - Cutting tolerance and potential for anaerobic digestion. *GCB Bioenergy*. doi:10.1111/gcbb.12330.

Lam MSJWQ, Martinez Y, Barbier O, Jauneau A, Pichon M (2013) Maize cell wall degradability, from whole plant to tissue level: different scales of complexity. *Maydica*, **58**, 103–110.

Lewandowski I, Heinz A (2003) Delayed harvest of miscanthus—influences on biomass quantity and quality and environmental impacts of energy production. *European Journal of Agronomy*, **19**, 45–63.

Lewandowski I, Kicherer A (1997) Combustion quality of biomass: practical relevance and experiments to modify the biomass quality of *Miscanthus* x *giganteus*. *European Journal of Agronomy*, **6**, 163–177.

Lewandowski I, Clifton-Brown JC, Scurlock JMO, Huisman W (2000) Miscanthus: European experience with a novel energy crop. *Biomass and Bioenergy*, **19**, 209–227.

Lewandowski I, Clifton-Brown JC, Andersson B *et al.* (2003a) Environment and harvest time affects the combustion qualities of Miscanthus genotypes. *Agronomy Journal*, **95**, 1274–1280.

Lewandowski I, Scurlock JMO, Lindvall E, Christou M (2003b) The development and current status of perennial rhizomatous grasses as energy crops in the US and Europe. *Biomass and Bioenergy*, **25**, 335–361.

Li X, Ximenes E, Kim Y, Slininger M, Meilan R, Ladisch M, Chapple C (2010) Lignin monomer composition affects *Arabidopsis* cell-wall degradability after liquid hot water pretreatment. *Biotechnology for Biofuels*, **3**, 27.

Li F, Ren S, Zhang W *et al.* (2013) Arabinose substitution degree in xylan positively affects lignocellulose enzymatic digestibility after various NaOH/H2SO4 pretreatments in Miscanthus. *Bioresource Technology*, **130**, 629–637.

Long SP, Beale CV, Farage PK (2001) Resource capture by miscanthus. In: *Miscanthus for Energy and Fibre* (eds Jones B, Walsh M), pp. 10–20. James & James (Science Publishers) Ltd., London.

Qin J, Yang Y, Jiang J, Yi Z, Xiao L, Ai X, Chen Z (2012) Comparison of lignocellulose composition in four major species of *Miscanthus*. *African Journal of Biotechnology*, **11**, 12529–12537.

Ralph J (2010) Hydroxycinnamates in lignification. *Phytochemistry Reviews*, **9**, 65–83.

Ralph J, Grabber JH, Hatfield RD (1995) Lignin-ferulate cross-links in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. *Carbohydrate Research*, **275**, 167–178.

Selig M, Weiss N, Ji Y (2008) Enzymatic saccharification of lignocellulosic biomass. *Laboratory Analytical Procedure*. report No. NREL/TP-510-42629, pp. 1–5. National Renewable Energy Laboratory, Golden, CO.

Studer MH, Demartini JD, Davis MF *et al.* (2011) Lignin content in natural Populus variants affects sugar release. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 6300–6305.

Tonn B, Thumm U, Lewandowski I, Claupein W (2012) Leaching of biomass from semi-natural grasslands–Effects on chemical composition and ash high-temperature behaviour. *Biomass and Bioenergy*, **36**, 390–403.

Torres AF, Van Der Weijde T, Dolstra O, Visser RGF, Trindade LM (2013) Effect of maize biomass composition on the optimization of dilute-acid pretreatments and enzymatic saccharification. *BioEnergy Research*, **6**, 1038–1051.

Torres A, Noordam-Boot CM, Dolstra O *et al.* (2014) Cell wall diversity in forage maize: genetic complexity and bioenergy potential. *BioEnergy Research*, **8**, 187–202.

Torres AF, Slegers PM, Noordam-Boot CMM *et al.* (2016) Maize feedstocks with improved digestibility reduce the costs and environmental impacts of biomass pretreatment and saccharification. *Biotechnology for Biofuels*, **9**, 63. doi:10.1186/s13068-016-0479-0.

Van Der Weijde T, Alvim Kamei CL, Torres AF, Vermerris W, Dolstra O, Visser RGF, Trindade LM (2013) The potential of C4 grasses for cellulosic biofuel production. *Frontiers in Plant Science*, **4**, 1–18.

Van Der Weijde T, Torres AF, Dolstra O, Dechesne A, Visser RGF, Trindade LM (2016) Impact of different lignin fractions on saccharification efficiency in diverse species of the bioenergy crop Miscanthus. *BioEnergy Research*, **9**, 146–156.

Van Soest PJ (1967) Development of a comprehensive system of feed analyses and its application to forages. *Journal of Animal Science*, **26**, 119–128.

Wyman CE (2007) What is (and is not) vital to advancing cellulosic ethanol. *Trends in Biotechnology*, **25**, 153–157.

Xu N, Zhang W, Ren S *et al.* (2012) Hemicelluloses negatively affect lignocellulose crystallinity for high biomass digestibility under NaOH and H2SO4 pretreatments in Miscanthus. *Biotechnology for Biofuels*, **5**, 58.

Yu P, Mckinnon JJ, Christensen DA (2005) Hydroxycinnamic acids and ferulic acid esterase in relation to biodegradation of complex plant cell walls. *Canadian Journal of Animal Science*, **85**, 255–267.

Zhao X, Zhang L, Liu D (2012) Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. *Biofuels, Bioproducts and Biorefining*, **6**, 465–482.

Zhao H, Li Q, He J, Yu J, Yang J, Liu C, Peng J (2014) Genotypic variation of cell wall composition and its conversion efficiency in *Miscanthus sinensis*, a potential biomass feedstock crop in China. *GCB Bioenergy*, **6**, 768–776.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Information on genotype backgrounds.